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CONTROL OF MOLTING IN MANDIBULATE AND CHELICERATE ARTHROPODS BY ECDYSONES

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Ecdysone and related steroids are the molting hormones of insects (*cf.* Novak, 1966; Kaplanis, Thompson, Robbins and Bryce, 1966; Kaplanis, Thompson, Yamamoto, Robbins and Louloudes, 1967; Thompson, Kaplanis, Robbins and Yamamoto, 1967; Williams, 1968). When 0.5 to 5 μg of these compounds are injected into isolated pupal abdomens of Cynthia moths, for example, they cause prompt development and the pupal abdomen molts into the corresponding fragment of an adult abdomen (Williams, 1968). The sequence of events triggered off by ecdysones in insects, which are collectively termed "molting," includes separation of the epidermis from the old cuticle (apolysis), the secretion of molting fluid, the secretion of a new cuticle, the digestion and resorption of part of the old cuticle, and the shedding of the old cuticle (ecdysis). Similar events occur in crustaceans during a normal molt cycle (Passano, 1960), but the control agents have not yet been identified. Evidence that ecdysones might be involved came from studies of Carlisle (1965) which showed that ecdysone extracts from different crustaceans and locusts caused molting in the crab *Carcinus maenas* from which Y-glands had been extirpated. Furthermore, ecdysones identical in their chemistry to those of insects were isolated from crustaceans (Hocks, Schulz and Karlson, 1967; Hampshire and Horn, 1966; Galbraith, Horn, Middleton and Hackney, 1968) and Lowe, Horn and Galbraith (1968) reported that injection of ecdysterone (= 20 hydroxy ecdysone or β-ecdysone) isolated from the crustacean, *Jasus lalandi*, caused shortening of the interecdysial period in eye-stalkless crayfish, *Procambarus simulans*. However, when we began these experiments, no reports existed of the successful induction of molting in arthropods other than insects by purified ecdysones.

The first example of ecdysone-induced molting in arthropods other than insects was the induction of molting in an isopod, *Armadillidium vulgare*, by ecdysterone (Krishnakumaran and Schneiderman, 1968, 1969). About nine days after receiving 150 μg/g of ecdysterone, the epidermis of the posterior parts of those isopods underwent apolysis and secreted a new cuticle and, two to three days later, underwent normal ecdysis. This was followed by secretion of a new cuticle and ecdysis of the anterior part, after which the animals resumed normal activity. The fact that both insects and crustaceans used ecdysones suggested that all mandibulate arthropods might use the same molecule for the control of molting. It was unclear, however, whether the other major subdivision of arthropods, the chelicerates, also use the ecdysones to control molting. The data in this report demonstrates that ecdysterone and several other ecdysones induce molting in crustaceans. These include

cyasterone, ponasterone A, α ecdysone, inokosterone and an ecdysone analogue, β SEA 1. The data also show that diverse chelicerate arthropods including spiders, tarantulas and horseshoe crabs can be caused to molt by the injection of ecdysterone. The results also demonstrate that injected ecdysones cause a similar pattern of cuticle formation and molting in crustaceans, insects and arachnids. The possible evolutionary significance of a common path for the chemical control of molting in arthropods is discussed.

A preliminary account of these results was published earlier (Krishnakumaran and Schneiderman, 1968). In addition, Wright (1969) has recently demonstrated that ecdysone causes molting in ticks.

Materials and Methods

Experimental animals

Two species of crayfish of the genus *Procambarus* were used. One was collected locally in northeastern Ohio and the other was purchased from Schettle Biologicals, Stillwater, Minnesota. The Ohio forms weighed between 3 to 12 grams while Minnesota forms weighed between 10 to 22 grams. The crayfish were maintained in the laboratory in shallow aquaria containing tapwater. The temperature and photoperiod were those of the laboratory (24–26° C and 12–15 hrs of light). Elodea served as food and also helped in oxygenation. In addition, the animals were fed meat once every two weeks. They survived for long periods under these conditions and several of them molted spontaneously in early summer and late fall. These ecdysed normally and survived the molt, provided that they were isolated from their cannibalistic neighbors when they were newly ecdysed. The crayfish were maintained in the laboratory for several days before they were used for the experiments. This helped in determining the normal rate of molting for that season of the year. In certain seasons (*e.g.*, spring), it was necessary to wait until the crayfish molted once in the laboratory before they could be used.

Another crustacean used in this series of experiments was the fiddler crab, *Uca pugilator*. Specimens of *U. pugilator* were collected off Woods Hole and shipped to Cleveland in August where they were maintained in sand troughs containing "instant ocean," an artificial seawater (Aquarium Systems, Inc., Cleveland, Ohio). They were fed periodically on crab meat and survived for several months. Crabs in the process of molting could be recognized by their pale carapace and lethargic movements, and were isolated individually in small transparent plastic containers containing sand and instant ocean. If such crabs failed to ecdyse or died within a week after isolation, they were peeled and examined to determine whether or not they had secreted a new cuticle.

Three different chelicerate arthropods were also used. The horseshoe crab, *Limulus polyphemus*, the spider, *Araneus cornutus*, and the tarantula, *Dugesiella hentzi*. Specimens of *L. polyphemus* were collected off Woods Hole, Massachusetts in July and were maintained either in running sea water tanks at the Marine Biological Laboratory there, or in an aquarium containing "instant ocean." Animals weighing 30 to 50 g each or 3 to 12 g each were used. No food was given during the experiment except for debris that might have been brought in by the running sea water and from the seaweed and colonial invertebrates present in the

aquarium. Animals were maintained at 19° C and received approximately 12 to 15 hrs of daylight. No effort was made to regulate photoperiod. Such animals survived several months under these conditions and several were still alive after a year in the artificial sea water.

The spider *Araneus* and the tarantula *Dugesiella* were obtained from Carolina Biological Supply, Inc. *Araneus* were collected in Oregon and were maintained individually in 30 ml plastic vials with screw caps. They weighed between 100 to 400 mg each and were sexually mature as evidenced by the production of egg cocoons. *Tenebrio* pupae were given as food weekly. The animals were kept in the dark at 18° C. These animals displayed no external morphological signs of molting prior to ecdysis. However, most of the spiders that ultimately secreted a new cuticle, stopped feeding and spun a molting pad which consisted of a few threads attached to the wall of the vial. Normally, these animals spin an orb web.

Tarantulas were maintained individually in glass tanks, the floor of which was covered with gravel. A cardboard tube served as a hiding place. They weighed between 8 to 12 g and both sexes were used. Live crickets and *Tenebrio* pupae were given as food weekly.

Hormones and other reagents

Ecdysterone and inokosterone were obtained from Rohto Pharmaceuticals, Osaka, Japan or Mann Research Laboratories, New York. α ecdysone was provided by Dr. P. Hocks, Schering, A. G. Berlin, ponasterone A was provided by Dr. John Pollard of Calbiochem. Cyasterone was provided by Professor C. M. Williams and came from the laboratory of Professor T. Takemoto, Tohoku University, Sendai, Japan. Drs. W. Robbins and Malcolm Thompson of the USDA at Beltsville, Maryland, provided the four ecdysone analogues: β SEA-1 (Δ^7-5β-cholestene-2β, 3β, 14α-triol-6-one), β SEA-4 (Δ^7-5β-cholestene-2β, 3β-diol-6-one), β SEA-12 (Δ^7-5β-sitostene-2β, 3β, 14α-triol-6-one) and α SEA-1 (Δ^7-5α-cholestene-2β, 3β, 14α-triol-6-one). Cholesterol and beta sitosterola were purchased from Nutritional Biochemicals, Cleveland, Ohio. Tritiated thymidine (Schwartz Bioresearch) was employed at a concentration of 1 mc/ml with a specific activity of 1.9 c/m Mole.

Ecdysterone and inokosterone were dissolved in insect Ringer (Ephrussi and Beadle, 1936), 10% ethanol or crustacean Ringer (Pantin's Ringer according to Marine Biological Laboratory Formulary, M.B.L., Woods Hole, Massachusetts) as the case may be. Cyasterone and ponasterone were dissolved in 10% ethanol, and α ecdysone in 20% ethanol. The ecdysone analogues were suspended in 20% ethanol, whereas the cholesterol and beta sitosterol were either dissolved in absolute ethanol or suspended in 50% ethanol.

Experimental procedures

Animals were anaesthetized in crushed ice and/or carbon dioxide. The materials were injected via a glass needle or 31 gauge steel needle using a Hamilton microliter syringe or a microburette. The volume of the material injected was usually less than 2 to 4% of the weight of the animal. Animals were kept for 2 to 4 hours at 5° C after injection before they were returned to the temperature

at which they were normally maintained. Tritiated thymidine was injected at a dose of 10 μc/g live weight of the animal. The isotope was administered at specific times after the injection of ecdysterone and was allowed to circulate for specific periods of time before the animals were killed. Animals to be killed were sliced in half and plunged immediately into Bouin's fluid and processed to make 5 to 6 μ thick paraffin sections. Autoradiographic methods were similar to those described earlier (see Krishnakumaran, Berry, Oberlander and Schneiderman, 1967).

Each experiment was repeated at least once, and in several cases two or three times. In all cases the results obtained were comparable and the results of typical experiments are shown in the tables.

Results

1. *Effects of ecdysterone on* Procambarus

In the first series of experiments, ecdysterone dissolved in Ringer solution was injected to a final concentration of 20 μg/g live weight. The data in Table I reveal that all the experimental animals molted within 10 days of the injection. The molt was abnormal, and in no case was there successful ecdysis and survival of the experimental animals. However, the animals did undergo apolysis and secreted a new cuticle which became obvious when the old cuticle was peeled away. All of the animals showed a swelling between the cephalothoracic shield and the abdomen. In a few cases the cephalothoracic shield separated from the new cuticle near its junction with the abdominal tergites.

Table I

Effect of ecdysterone on molting in the crayfish, Procambarus *sp.*

Chemical and dose	Number of animals	Per cent that molted within a month
Uninjected control	10	10
Ethanol 10% 4 μl/g	10	0
Cholesterol 20 μg/g	10	0
Ecdysterone 20 μg/g	10	100*
10 μg/g	10	100*
6 μg/g	10	100*
3 μg/g	10	100†

Animals weighed between 12 and 20 grams each.
* Molted within 10 days after injection.
† Molted within 14 days after injection.

In insects it is known that high doses of ecdysones are pathological and frequently cause the death of the injected animals (Kobayashi, Takemoto, Ogawa and Nishimoto, 1967; Williams, 1968). Presuming that the mortality observed in the preceding experiment may have been the result of a pathologically high dose of ecdysterone, lower concentrations of ecdysterone were injected. In one series of experiments we injected 3, 6, or 10 μg/g ecdysterone. Table I shows that even 3 μg/g of ecdysterone induced molting in *Procambarus*. However, even after such a low dose, almost all of the experimental animals died during ecdysis. Only two

of the crayfish which received 3 μg/g ecdysterone completed ecdysis and survived. Even these were able to shed spontaneously only the caphalothoracic shield, and the remaining cuticle had to be peeled away.

Although between 3 and 20 μg/g of ecdysterone were effective in inducing molting processes, there were distinct differences in the response to low and high doses. Thus an injection of 6, 10, or 20 μg/g of ecdysterone resulted in apolysis and secretion of a new cuticle within ten days, whereas animals injected with 3 μg/g took about fourteen days to secrete a new cuticle. Crayfish receiving lower doses of ecdysterone also differed in the size of the gastroliths formed and the extent of digestion of the old cuticle. Gastroliths were best developed and the digestion of old cuticle most pronounced in crayfish that received 3 μg/g ecdysterone. The gross structure and histochemistry of the cuticles deposited in response to different doses of ecdysterone were identical, but certain morphological features of the cuticle were affected by the dose. Thus, the newly-deposited cuticle was similar to normal cuticle and contained a non-chitinous epicuticle, a chitinous, lamellated endocuticle and polyphenolase. However, animals that received 20 μg/g ecdysterone secreted a much thinner new cuticle than those that received lower doses of the hormone. Also, the bristles and hairs on the uropods and the branchial gills were shorter and ill-formed in all experimental animals except those that received only 3 μg/g ecdysterone.

An additional effect of high doses of ecdysterone was its inhibition of regeneration of appendages. Crayfish that had lost some of their appendages (usually walking legs) several weeks before hormone treatment, were injected with 20 μg/g ecdysterone. These animals molted promptly, between 7 and 10 days after they received the hormone, without regenerating the lost appendages. However, similar animals that received 2 to 3 μg/g ecdysterone formed a small regenerate. In contrast, crayfish whose appendages were amputed only 7 to 10 days prior to the injection of ecdysterone failed to regenerate even after treatment with 2 to 3 μg/g ecdysterone. These results are reminiscent of the situation in the wax moth *Galleria* (Madhavan and Schneiderman, 1969) where regeneration of imaginal wing discs in the last larval instar is promoted by low doses of ecdysone, but fails to occur after injecting high doses. In the crayfish, as in *Galleria*, ecdysone may be necessary for regeneration, but when applied in high doses it provokes molting so promptly that there is insufficient time for the cell divisions necessary for regeneration.

2. *Histological and autoradiographic studies of the crayfish epidermis under the influence of ecdysterone*

To determine when the various events of the molt cycle occurred in the epidermis of the crayfish under the influence of ecdysterone, *Procambarus* was injected with 2 μg/g ecdysterone, and three animals were killed and examined at 4, 24, 48, 96, and 144 hours after the injection of the hormone. Animals were processed for histological study and 4 to 5 micron thick paraffin sections were prepared and stained with Mallory's triple stain or Meyer's haemalum and eosin. A study of these sections revealed that the old cuticle had apolysed from the epidermis about 48 hrs after the injection of hormone. By 96 hrs after injecting the hormone, a new cuticle, approximately 8 to 10 μ thick in the tergite region, had already been secreted.

At this time the nuclei, which began to enlarge at the time of apolysis, were greatly enlarged. Associated with this nuclear enlargement was an increase in the size and basophilia of the epidermal cells. Events which occur after 96 hrs are difficult to analyze in the present experiments because they may have been associated with the pathological changes connected with the imminent death of the animals. Six days after the injection of ecdysterone, the epidermis in some of the crayfish showed further changes, such as a decrease in cytoplasmic volume. They resembled to some extent intermolt epidermis, except that in the normal intermolt animal the nuclei of epidermal cells are compact and lack chromatin granules.

Analysis of DNA synthesis in epidermis was undertaken next. For this purpose crayfish were injected with ecdysterone at a dose of 2 μg/g and immediately thereafter, or 4, 24, 48, or 96 hrs later, tritiated thymidine (10 μc/g) was injected. Individuals were killed both 2 and 24 hrs after the injection of isotope. None of

TABLE II

Effect of ecdysone analogues and steroids on molting in the crayfish, Procambarus *sp.*

Chemical and dose	Number of animals	Per cent that molted
10% ethanol 3 μl/g	10	0
Ecdysterone 6 μg/g	10	100
Inokosterone 6 μg/g	6	100
Cholesterol 6 μg/g	16	0
Cholesterol 20 μg/g	15	0
β SEA-1 50 μg/g	6	50
α SEA-1 50 μg/g	6	0
β SEA-4 50 μg/g	6	0
β SEA-12 50 μg/g	6	0
β Sitosterol 20 μg/g	6	0

Animals weighed between 12 and 20 grams each.

the epidermal cells incorporated tritiated thymidine into their nuclei, although blood cells engaged in extensive DNA synthesis during this period, and many well-labelled blood cells were seen. Apparently, the ecdysterone at the levels used in these experiments does not induce DNA synthesis in the epidermis of these crayfish, although it induces molting. It is noteworthy also that muscles, nerve cells, and connective tissue failed to synthesize DNA.

3. *The specificity of ecdysterone in inducing molting*

Does ecdysterone induce molting in these crayfish because it is either the normal molting hormone or an analogue of the normal hormone, or, is it possible that its effects represent a nonspecific pharmacological action of steroids? To test this possibility, we injected crayfish with various sterols, such as cholesterol and beta sitosterol or ecdysone analogues such as β SEA-1, α SEA-1, β SEA-4, and β SEA-12. These agents have very little or no ecdysone effects in insects. The results are recorded in Table II. Crayfish injected with 6 or 20 μg/g cholesterol or 20 μg/g sitosterol or 50 μg/g α SEA-1, β SEA-4, or β SEA-12 did not molt. These data suggest that the effects of ecdysterone are not due to a nonspecific pharma-

cological effect of steroids. Even chemicals closely related to the ecdysones, such as α SEA-1, β SEA-4 and β SEA-12, do not induce molting (see Table II).

The only chemical that showed any molt-inducing effect in *Procambarus* was β SEA-1, which is structurally similar to the ecdysones except for the absence of hydroxyl groups on the side chain. This substance has ecdysone-like effects on the house fly and *Calliphora* (Robbins, personal communication). In *Procambarus* it caused molting in 50% of the crayfish into which it was injected. These animals developed fully-formed gastroliths, more-or-less completely digested their old cuticle and underwent spontaneous partial ecdysis, after which they died. Unlike ecdysterone, which induced prompt molting, β SEA-1 caused animals to molt 3 to 4 weeks after they received an injection.

4. *Effects of phytoecdysones on* Procambarus

From the preceding experiments it appears that ecdysterone induces molting in crayfish by specific hormonal action rather than by some nonspecific pharmacological effect. Is this activity limited to ecdysterone which normally occurs in crustaceans, or do the other zooecdysones and phytoecdysones that cause molting in insects also induce molting in crayfish? To test this, α ecdysone, inokosterone, ponasterone A and cyasterone were injected into *Procambarus*. An injection of 3 μg/g of any of these ecdysones induced molting in these crayfish. But as Table III shows, there were differences in the percentage of animals that responded and the time required for a response. Cyasterone was the least effective and induced molting in only 30% of the animals. In contrast, ecdysterone was the most effective and induced molting in all of the experimental animals. Inokosterone, α ecdysone and ponasterone were intermediate in effectiveness with 86%, 66% and 46% of the animals molting, respectively. When higher doses of cyasterone (6 and 10 μg/g) were injected, all of the treated crayfish molted, indicating that the response was dose-dependent.

TABLE III

Effects of different ecdysones on molting in the crayfish, Procambarus *sp.*

Treatment	Number of animals	Per cent that molted during the period indicated (approximate)			Cumulative % of crayfish that molted during the 21 day period
		7–10 days	10–14 days	14–21 days	
Uninjected controls	25	0	0	0	0
Controls injected with 10% ethanol 3 μl/g	10	0	0	0	0
Ecdysterone 3 μg/g	7	14	86	0	100
α Ecdysone 3 μg/g	15	56	7	7	66
Inokosterone 3 μg/g	14	21	56	7	86
Ponasterone A 3 μg/g	15	14	35	0	46
Cyasterone* 3 μg/g	10	10	10	10	30

Animals weighed 3 to 20 grams.
Most of the animals died in the process of molting.
* Higher doses, such as 6 μg/g and 10 μg/g, induced molting in 100% of experimental animals.

Another distinguishing feature of the different ecdysones was the interval between the time of injection of hormone and the induction of the molt. An injection of 3 μg/g of α ecdysone caused 56% of the animals to molt between 7 and 10 days, whereas in the case of ponasterone, inokosterone and ecdysterone, most of the animals molted between 10 and 14 days after injection. The reasons for this difference are not obvious; it is possible that α ecdysone is the true hormone with a short half-life, while other ecdysones may be less effective, but more stable, degradation products or analogues of α ecdysone (*cf.* King and Siddall, 1969).

Almost all of the crayfish that had been induced to molt experimentally, died after secreting a new cuticle. In an effort to increase their survival, we removed their eye stalks. The rationale behind this maneuver is the fact that eye stalks are known to produce and store a molt-inhibiting agent (*cf.* review by Passano, 1960). The site of action of this agent is not known, but if it acted upon epidermal cells, then its removal might permit better survival after ecdysis. Eye stalks were removed the day prior to, or immediately after, the injection of 3 μg/g of ecdysterone. However, survival after the molt was not increased.

5. *Effects of ecdysterone on* Uca pugilator

Another experiment tested the effects of ecdysterone on a marine crustacean, *Uca pugilator,* a semi-terrestrial fiddler crab. Fiddler crabs received 20 μg/g of ecdysterone dissolved in 4 μl of Ringer solution. Controls received 4 μl of Ringer solution or no injection. The results, recorded in Table IV, show that 90% of

TABLE IV

Effect of ecdysterone on the induction of molting in the fiddler crab, Uca pugilator

Treatment	Number of animals	Per cent that spontaneously ecdysed during the specific period (*) or revealed a new cuticle after peeling			Cumulative % of crabs that molted during the 30 day period
		0–10 days	10–20 days	20–30 days	
Uninjected controls	15	0	7*	7*	14
Controls injected with 4 μl/g Ringer	30	10*	3*	3*	16
Experimentals injected with 20 μg/g ecdysterone in 4 μl Ringer	30	0	83	7	90

the experimental animals molted within 30 days, whereas only 16% of the injected controls and 14% of the uninjected controls molted during this period. Unlike the controls which underwent normal ecdysis, the experimental animals never underwent spontaneous ecdysis, although they showed apolysis, deposition of a new cuticle, secretion of molting fluid and partial resorption of the old cuticle. The experimental animals that had deposited a new cuticle could easily be recognized by their pale color and lethargic movements. When such crabs were peeled, their new cuticle was revealed, but they immediately died. Even if left for 10 days after the first appearance of pale color, they failed to undergo spontaneous ecdysis. Another feature of the experimental molt was that a large percentage of the animals responded within the same short span of time; namely, 14 to 18 days after injection of ecdysterone.

The cuticle formed in response to the injection of ecdysterone was similar in general appearance to normal cuticle and possessed epicuticle, a lamellated endocuticle and polyphenolase. However, experimentally-induced cuticle differed from the normal cuticle in several respects. In general, the induced cuticle was thinner than the normal cuticle. This may be in part the result of premature death, but appeared to be associated more with the artificial induction of molting. Another conspicuous difference was in the nature of the tubercles on the lateral borders of the dactylus and claw and the anterior ventral margins of the carapace. The tubercles in the experimental animals were much smaller and ill-formed. However, their distribution and orientation was identical to the pattern found in the old cuticle. Similarly, the bristles and sensory hairs at the borders of the appendages and on the general cuticular surface were short and ill-formed. In normal animals, the cuticle on the two sides of the maxilliped differ in thickness: the external surface has a thick cuticle (20 to 25 μ), whereas the internal surface has a thin cuticle (4 to 6 μ). In experimentally-treated animals the cuticle on both sides of the maxilliped is of the same thickness (4 to 6 μ). In addition, in normal animals, the external surface of the maxilliped bears tubercles, while in the experimentally-treated animals, there were either no tubercles or small tubercles.

TABLE V

Effect of ecdysterone on molting in Araneus cornutus

Treatment	Number of animals	Per cent that molted during the period indicated				Cumulative % of spiders that molted during the 29-day period
		0–8 days	8–15 days	15–22 days	22–29 days	
Controls injected with 4 μl of Ringer	24	8*	0	4*	0	13
Experimentals injected with 20 μg of Ecdysterone in 4 μl Ringer	29	7*	28	14	14	62

* Survived after spontaneous ecdysis. Others were peeled to determine whether they had molted.

Control and experimental groups contained 29 and 30 spiders each, respectively. One of the experimentals and 5 of the controls died within the first three days after injection and are not included in the data. Sixteen controls and six experimentals survived for the duration of the experiment but did not show any signs of molting.

6. *Effects of ecdysterone on* Araneus cornutus

In the first series of experiments with the spider *Araneus,* animals were chilled on crushed ice and injected with 20 μg/animal ecdysterone in 4 μl of Ringers. The animals weighed approximately 150 mg, and thus the average dose of hormone was about 130 μg/g. Controls received 4 μl of Ringer solution. Results reported in Table V show that 62% of the experimentals molted in the 4-week period after injection, whereas only 13% of the controls molted. Unlike the controls which survived spontaneous ecdysis and continued normal life, the experimentals underwent apolysis and deposited a new cuticle, but then died. This was true of all the experimentally-treated animals that molted except for two that molted within

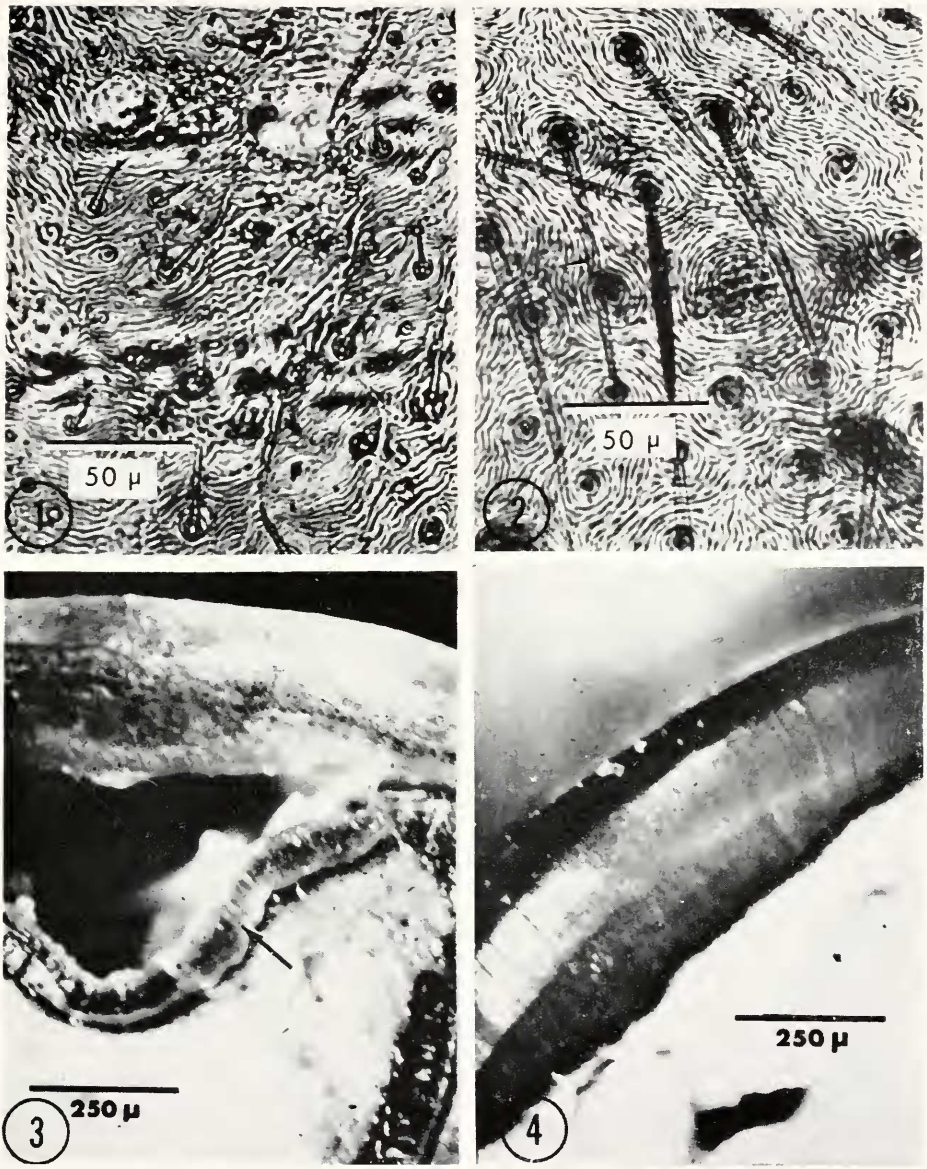

FIGURE 1. Surface view of cuticle from opisthosomal tergite in the spider, *Araneus cornutus* induced to molt by injection of ecdysterone. Note the short bristles and the inconspicuous ridges.

FIGURE 2. Same view as above from a control spider. The bristles are much longer and the ridges are conspicuous.

FIGURE 3. Cross section of telson from ecdysterone-injected *Limulus* showing the newly formed cuticle (arrow) inside the old cuticle.

FIGURE 4. Cross section of telson from control *Limulus* injected with Ringer.

the first eight days after receiving the injection of ecdysterone. Probably these had already begun spontaneous ecdysis before the hormone injection.

Frequently it was possible to recognize spiders that were about to secrete a new cuticle and molt by the fact that they ceased to feed and spun a molting pad rather than the normal orb web. Control spiders underwent spontaneous ecdysis, usually within a week after they ceased feeding and spun a molting pad, whereas the experimental spiders died. When the dead, hormone-treated spiders were peeled, it became evident that they had secreted a new cuticle. However, although the cuticle secreted after the injection of ecdysterone resembled normal cuticle in the arrangement of the basic cuticular layers and its staining properties, it was abnormal, particularly in its surface pattern and in the morphology of spines and bristles. While normal cuticle bears conspicuous ridges on its outer surface, in the experimental cuticles, the ridges were either shallow or absent. Spines which are long in normal cuticles were much reduced in length in the experimentals and frequently did not rise above the surface of the cuticle. However, the sockets bearing these spines appeared to be normal in number and distribution in experimental animals (Figs. 1 and 2).

In a second series of experiments, ecdysterone was dissolved in absolute methanol to a final concentration of 5 μg/μl and applied topically to the surface of the abdomen. Each spider received either 4 μl of this methanolic solution or was dipped in a methanolic solution. The controls received either the same amount of absolute methanol or were dipped in methanol. Whereas 50% of the ecdysterone-treated spiders molted in a three week period, only 15% of the controls molted during this period. However, the mortality was much higher than after injection of ecdysterone in Ringer solution.

7. *Effects of ecdysterone on* Dugesiella hentzi

The primitive tarantula spider, *Dugesiella,* was used in the following experiments. A group of four tarantulas (2 males and 2 females), weighing between 8 and 12 g each, were injected with 200 μg of ecdysterone in 40 μl of Ringer solution, an effective dose of 16 to 25 μg/g. Controls (2 males and 2 females) received 40 μl of Ringer solution. Both groups remained active and fed normally, but neither the experimentals nor the controls molted during the 60-day period they were observed. Two months after the initial injections, three of the experimental animals were injected with a larger dose of ecdysterone—50 μg/g—and the fourth was injected with 100 μg/g. Controls received corresponding amounts of Ringer solution. Seven days after receiving the second injection of ecdysterone, one of the experimental animals, which received 50 μg/g, died. Dissection revealed that the animal had undergone apolysis but there were no signs of a new cuticle. Twenty days after the second hormone injection, another of the tarantulas underwent spontaneous ecdysis and survived. It secreted a normal cuticle replete with spines and bristles. (This same animal underwent a normal, uninduced, spontaneous molt ten months later and is alive at the time of writing). Between 22 and 28 days after the second hormone injection, the other two hormone-treated animals also molted. These showed both apolysis and the secretion of a new cuticle, but did not undergo spontaneous ecdysis. Like the old cuticle, the new cuticle possessed a distinctive fuchsinophilic epicuticle and an aniline blue-stained

lamellated endocuticle. However, it differed from the normal cuticle in the absence of bristles and spines. The controls survived for several months without molting. One of these was killed two months after the second injection of Ringers and there were no signs of initiation of a molt. Since the molts in all four experimental animals were induced in September, three months after the normal natural period of molting in these animals, and since none of the four controls molted spontaneously during this period, it seems clear that the injection of ecdysterone induced the molt.

8. *Attempts to demonstrate effects of ecdysterone on isolated abdomens of spiders*

One sure way to determine whether ecdysterone is responsible for the induction of molting in insects has been the testing of the hormones on isolated abdomens which are devoid of the major endocrine glands. The morphology of spiders appeared to make them particularly amenable to such a surgical maneuver. Although we do not know the source of the molting hormone in the spiders, we assumed that the glands responsible for molting are not located in the abdomen. With this gratuitous assumption in mind, we prepared a number of isolated abdomens of *Araneus* and *Dugesiella* and some other common spiders by ligating the narrow waist-like opisthosoma. The anterior halves of these animals were excised and, after applying penicillin, streptomycin and phenylthiourea, the wound was sealed with paraffin. These isolated abdomens survived for a week to ten days. However, injection of 20 μg of ecdysterone failed to cause molting. These unsuccessful results are

TABLE VI

Effects of ecdysterone on molting in Limulus polyphemus

Treatment	Number of animals	Per cent showing signs of molt within one month	Remarks
Uninjected controls	50	2	Only apolysis
Controls injected with 8 μl of Crustacean Ringer	6	0	—
Experimental injected with 40 μg/g ecdysterone in 8 μl Ringer	5	100	Secreted new cuticle but had to be peeled

Animals weighed between 35 and 50 grams each and were kept in running sea water.

reported only because this may be the first report of such surgical operations on spiders and may prove useful for some other study. The negative results we obtained may reflect the fact that we performed only a limited number of operations.

9. *Effects of ecdysterone on* Limulus polyphemus

The horseshoe crab, *Limulus* is a relict merostomate arachnoid, only remotely related to modern arachnids, such as spiders and scorpions. Living genera of horseshoe crabs resemble closely the ancient genus *Paleolimulus* which lived some 200,000,000 years ago in the Permian. Their closest relatives are thought to be the extinct eurypterids and trilobites. Large specimens of *Limulus* which weighed 35 to 50 grams each, were injected with 40 μg/g ecdysterone dissolved in crustacean Ringer. Controls were either injected with a corresponding amount of Ringer or

were uninjected. The results recorded in Table VI show that all experimentals molted. Only one of the fifty uninjected controls and none of six injected controls showed any signs of molting. Even the one control animal that showed signs of molting, underwent only partial apolysis. In *Limulus*, as in the other animals studied in this report, the experimentally-induced molt was different from a normal molt. The animals underwent apolysis and became paler in color but they never spontaneously shed their cuticles. Microscopic examination of such animals after peeling the old cuticle confirmed that the new cuticle had the same cuticular layers as the old cuticle (Figs. 3 and 4). Treatment of a second batch of smaller specimens of *Limulus* (3 to 10 grams) gave similar results.

These two series of experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, where the specimens of *Limulus* were kept in tanks of running sea water. When the experiments were repeated in the laboratory at Cleveland, Ohio, using artificial sea water in a circulating system, they were unsuccessful. However, all 25 of the ecdysterone-injected specimens of *L. polyphemus* died within four weeks, whereas 60% of the 25 controls injected with Ringer survived for more than four months. None of the controls or the experimental animals deposited a new cuticle.

Discussion

1. *Ecdysones as the true molting hormones of all arthropods*

The data show that ecdysterone causes molting in diverse chelicerate and mandibulate arthropods. The effects appear to be true hormonal effects and not nonspecific effects of steroids. For example, less than 3 μg/g of ecdysterone caused molting in crayfish, whereas doses up to 50 μg/g of steroids such as cholesterol, beta sitosterola and the ecdysone analogues (β SEA-4, β SEA-12), which are hormonally inactive in house fly and *Calliphora* assays, have no effect in crayfish. A second reason for believing that the ecdysones are the true molting hormones of these arthropods is the occurrence of α ecdysone, deoxyecdysone, ecdysterone and at least two other ecdysones in crustacean (Galbraith *et al.*, 1968; King and Siddall, 1969; Faux, Horn, Middleton, Fales and Lowe, 1969). Although their presence in chelicerate arthropods has not been uncovered thus far, it seems likely that ecdysones occur in these arthropods as well. Thirdly, the dose required to induce molting in insects (Williams, 1968; Krishnakumaran, Granger and Schneiderman, 1970) and crustaceans (present results) is in the same physiological range.

A fourth line of evidence that supports the view that ecdysones are the natural molting hormones of arthropods other than insects is the abnormality of the cuticle deposited in response to ecdysone treatment. The experimentally-induced cuticles of spiders and crabs show several features which are similar to the pathological effects of high doses of ecdysones in insects. Such pathological effects in insects have been termed "hyperecdysonism" by Williams (1968) and appear to represent the first example of hyperhormonism in invertebrates. In Lepidoptera, these features include short, ill-formed bristles and scales, a decreased number of bristles and scales, an abnormal cuticular texture, and have been attributed to a telescoping of the normal sequence of synthetic events in which epidermal cells engage during the process of cuticle deposition. In the present experiments similar abnormalities

were found in spiders (fewer and shorter hairs and bristles) and in fiddler crabs (reduced size of knobs on dactylus and on the anterior ventral surface of the carapace). These abnormalities appear to be caused by the abnormal dosage and timing of the application of the hormones: Normally ecdysone is released gradually, but in these experiments it was applied all at once. It seems that such "hyperecdysonic" effects would be expected only if ecdysterone were either a true natural molting hormone or of similar structure to the true molting hormone. The following facts also support this opinion.

If ecdysone caused some general pathological effect which resulted in abnormal cuticles, it might be effective even after the process of molting had begun. This is not the case. Injection of a high dose of ecydysterone after the initiation of molting has no "hyperecdysonic" effects in insects. The same appears to be true of the spiders which molted during the first eight days after injection of the ecdysterone. These spiders ecdysed spontaneously and produced a normal-looking cuticle, apparently because molting had been initiated prior to the injection. Thus, it appears reasonable to presume that the action of ecdysterone in the crayfish, and possibly in the other arthropods, is a true hormonal action.

What are the targets of ecdysone? In insects the targets of ecdysone include the epidermis (Wigglesworth, 1957) midgut (Piepho, Holz and Jung, 1964) nervous system (Pipa, 1969), imaginal discs (Madhavan and Schneiderman, 1969), several internal organs (Sehnal and Schneiderman, 1970) and sometimes the ecdysial glands (Schneiderman and Gilbert, 1964). The target tissues of ecdysones in other arthropods are probably the same, but certainly include the epidermis, for the following reasons. It is unlikely that the ecdysones cause molting in these diverse arthropods by activating the animal's own ecdysial glands, for if this were the case, one would not expect abnormal cuticles. When the ecdysial glands of insects are activated, by whatever means, normal cuticles are produced, provided one does not inject excess amounts of ecdysones. The abnormalities produced in the cuticle of these diverse arthropods by excessive amounts of ecdysone, suggest a direct action of the ecdysones on the epidermal cells. Apparently, at least the chitogenous epithelium of most arthropods is capable of responding to insect ecdysones. This implies that the final common path of the control of molting in most arthropods is the same, involving an ecdysone or ecdysone-like molecules and the associated receptor sites in the epidermal cells. Although definitive evidence for true homology must await the identification of the ecdysial glands in, and the isolation of ecdysones from chelicerate arthropods, the evidence presented in this report points to such homology.

The nature of the primary action of ecdysones on insect epidermal cells remains to be identified (*cf.* discussion in Krishnakumaran *et al.*, 1967). However, the results of the autoradiographic experiments on crustaceans reported here, which demonstrate that the epidermal cells of crayfish can secrete a new cuticle without first engaging in DNA replication, emphasize that the fundamental role of ecdysones as molting hormones may be uncoupled from any role they have have as growth hormones. Similar molts without DNA replication have been observed in adult insects (Krishnakumaran and Schneiderman, 1964; Krishnakumaran *et al.*, 1967) and in insects treated with high doses of ecdysones (Krishnakumaran, Granger and Schneiderman, 1970). It remains to be demonstrated when DNA replication normally occurs in the interecdysial period of crustaceans.

It is of interest that ecdysterone caused behavioral effects in addition to its effects on the cuticle. Spiders repsonded to ecdysterone by spinning a molting pad before any obvious changes in the cuticle were evident. Whether these are direct effects on the nervous system, analogous to those caused by sex hormones in vertebrates, or some indirect effects remains to be proven.

2. Relative activities of different ecdysones

In insects and in crayfish the active ecdysones were effective at doses varying between 0.5 to 5 μg/g live weight. In chelicerate arthropods, higher doses appeared to be necessary. Injections of about 40 to 50 μg/g were required to initiate molting in tarantulas and in *Limulus*. Perhaps the specific ecdysones employed by chelicerates are different from their counterparts in insects and crustaceans.

From a study of the relative activities of the five different ecdysones, it appears that ecdysterone is the most active in crustaceans, followed by inokosterone, α ecdysone and ponasterone A, with cyasterone being the least active. This contrasts with observations on some insects where cyasterone is among the most active of the ecdysones. For example, in the lepidopteran, *Samia cynthia*, the relative activities of the ecdysones are: cyasterone > ponasterone A > α ecdysone > ecdysterone > inokosterone (Williams, 1968). It is of interest also that β SEA-1, which is the least active of the active ecdysone analogues in insects, is also the least active ecdysone for crustaceans.

From the analysis of all the dose-effect data presently available to us, it appears that almost all of the ecdysones which are active in one group of arthropods will have some activity in other groups. Whether injected ecdysones actually affect target cells themselves, or are metabolically converted into other ecdysones which affect target cells, is unknown. Indeed, even in insects, the role of the interconversion of the several ecdysones is still unclear (*cf.*, for example, King and Siddall, 1969).

3. Survival after experimental molt

Insects which are caused to molt by injections of ecdysones commonly fail to survive. This is true also of most of the other arthropods examined here. In fact, only two each of the crayfish and spiders and one of the tarantulas which were caused to molt by ecdysones, survived for long periods after the molt. It is possible that the experimentally-induced molts caused death because of hyperecdysonism or because of the absence of certain necessary preparations for molting controlled by other hormones (such as the brain).

What are the probable causes of death in ecdysone-induced molts? In insects abnormal molting results in a defective cuticle in which wax layers may be incomplete and animals die because of desiccation (*cf.* review by Schneiderman, Krishnakumaran, Bryant and Sehnal, 1969). In other arthropods, defects in cuticle may have manifold effects. In crayfish, such defects may decrease their waterproofing and make them subject to dilution by fresh water. Both in spiders and in crustaceans, the presence of the old cuticle plus molting fluid over the book lungs or gills will decrease the effectiveness of these respiratory organs by increasing the distance across which gases must diffuse. Undoubtedly, there are other causes

of death, but it appears likely that many are associated with cuticle. Ecdysones themselves do not appear to be toxic unless they cause molting, and animals which received injections of ecdysone rarely died except in the process of molting.

The only crayfish that survived were those that received a low dose of ecdysterone. Perhaps this activated their own ecdysial glands, or the dose was in the physiological range. The crayfish that died showed abnormal calcium resorption as indicated by poor formation of gastroliths and incomplete resorption of the old cuticle. These crayfish never shed their cuticles, possibly due to a failure in the absorption of water which normally precedes ecdysis.

These results are in marked contrast to those we obtained with terrestrial isopods in which normal molting was induced in more than half the animals by injecting ecdysterone (Krishnakumaran and Schneiderman, 1969). What are the reasons for the survival of these isopods and the death of other hormone-treated arthropods? One reason may be the peculiar way in which isopods molt. Posterior and anterior halves of these pill bugs undergo ecdysis consecutively, 3 to 4 days apart. This may involve either (a) a mechanism to remain insensitive to high levels of ecdysones circulating in the hemocoel, or (b) a controlling device that regulates the time of response of the chitogenous epithelium or (c) a mechanism to inactivate the excess ecdysone or (d) a combination of the above. Such a mechanism would prevent any hyperecdysonic effects and thus permit better survival. Another possibility is that the ecdysone activates the animals' own ecdysial glands.

4. *Phylogenetic considerations*

The fact that the ecdysones are capable of inducing molting, not only in insects but also in diverse crustaceans and chelicerates, suggests close similarity or even identity of the mechanisms that control molting in arthropods. If this proves to be true, it may throw some light on the phylogeny of the arthropods. The similarity in the chemistry of the molting hormone, and by implication the receptor sites in the chitogenous epithelium, strongly suggests the common ancestry of all arthropods. Manton (1964) and earlier Tiegs and Manton (1958) contend that the arthropods are polyphyletic in origin. They argue that the similarities in the structure and chemistry of the cuticle (see Richards, 1951; Krishnakumaran, 1961) independently evolved by convergence. The fact that the mechanisms controlling secretion of the cuticle are also homologous, makes such an argument unlikely. The convergence hypothesis becomes even less tenable when one adds the fact that the ecdysial glands of both insects and crustaceans have similar origins from the ectoderm of the embryonic cephalic region (see Jenkin, 1962; Herman, 1967). We have no knowledge of the location, structure, and origin of the ecdysial glands in the chelicerates. Should the ecdysial glands of chelicerates prove to be of epidermal origin and arise from the prosomic region, it would establish beyond all reasonable doubt that the arthropods are truly a homogeneous group with a monophyletic origin.

However, if it turns out that ecdysones are present and function in a wide variety of invertebrates such as annelids, nematodes, priapulids and related aberrant schizocoelic groups, then it is still possible that different arthropod groups may have evolved independently from several diverse post-annelid, prearthropod

ancestors. It will be of interest to investigate the distribution and effects of ecdysones in annelids and other groups.

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Summary

1. The ability of ecdysones to induce molting in arthropods other than insects was examined in representatives of both the mandibulate and chelicerate subphyla.

2. All five ecdysones tested caused molting in the fresh-water crayfish, *Procambarus*. Their relative activities were: ecdysterone > inokosterone > α ecdysone > ponasterone A > cyasterone. Doses as low as 3 μg/g of ecdysterone caused 100% of all test crayfish to undergo apolysis and secrete a new cuticle within 14 days after injection, but only in a few cases did the animals shed their old cuticles spontaneously after experimental treatment. At higher doses the new cuticle was thinner than normal and had abnormal bristles. The stimulation of molting was specific for ecdysones and was not copied by a variety of ecdysone analogues or other steroids.

3. Histological and autoradiographic studies revealed that ecdysterone at the levels used in these experiments caused molting in crayfish without DNA replication.

4. Ecdysterone also caused molting in the marine fiddler crab, *Uca pugilator*.

5. Ecdysterone caused molting in several chelicerate arthropods including the spider, *Araneus cornutus*, the tarantula, *Dugesiella hentzii* and in the horseshoe crab, *Limulus polyphemus*, which is among the most primitive of all living arthropods.

6. In spiders the ecdysone caused behavioral effects before any obvious changes in the cuticle were evident.

7. In almost all cases, molts induced by ecdysone were characterized by abnormal cuticles similar to those produced by injections of ecdysones in insects, a result which suggests a direct action of ecdysone on the epidermal cells. Most of the experimental animals failed to survive the molt and few underwent spontaneous ecdysis. These effects probably result from the abnormal delivery of a large amount of hormones in one dose, in contrast to the gradual release of hormone *in situ*.

8. From an analysis of all of the dose-effect data, it is concluded that almost all of the ecdysones which are active in one group of arthropods will have some activity in other groups. Since spiders and horseshoe crabs require doses about ten times as high as those needed for mandibulate arthropods, the specific ecdysones employed by the chelicerates may differ from their counterparts in insects and crustaceans.

9. The evidence suggests that ecdysones are the normal molting hormones of all arthropods and supports the view that arthropods have a common ancestry and are not a polyphyletic group.

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